(12) UK Patent Application (19) GB (11) 2 217 600 (13) A

(43) Date of A publication 01.11.1989

(21) Application No 8907914.9

(22) Date of filing 07.04.1989

(30) Priority data
(31) 63086693 (32) 08.04.1988 (33) JP
01016759 13.01.1989

(51) INT CL'
A61K 39/00 39/155 39/165 39/20

(52) UK CL (Edition J)
A5B BAA B112 B113 B114 B120 B135

(56) Documents cited
GB 1548787 A EP 0175841 A2 EP 0156633 A1

(58) Field of search
UK CL (Edition J) A5B BAA
INT CL' A61K
WPI (DIALOG) Online

(71) Applicants
National Insitute of Health

(Incorporated in Japan)

2-10-35 Kamiosaki, Shinagawa-ku, Tokyo, Japan

The Kitasato Institute

(Incorporated in Japan)

5-9-1 Shirokane, Minato-ku, Tokyo, Japan

(72) Inventors
Shinichi Tamura
Takeshi Kurata
Chikara Aizawa
Takashi Nagamine

(74) Agent and/or Address for Service
J A Kemp and Co
14 South Square, Gray's Inn, London, WC1R 5EU, United Kingdom

# (54) Vaccine preparation

(57) A vaccine preparation comprising a vaccine and a toxin or a subunit thereof. The vaccine may be influenza vaccine, pertussis vaccine, Japanese encephalitis vaccine, a mixture of pertussis, diphtheria and tetanus toxoid, hepatitis B vaccine, Rota vaccine, measles vaccine, rubella vaccine, mumps vaccine or mycoplasma vaccine. The toxin is preferably a bacterial toxin such as cholera toxin, staphylococcal or S-hemolysin, vibrio thermostable direct hemolysin, pertussis toxin or E coli heat-labile toxin.

GB 2 217 600 A

FIG. 1A

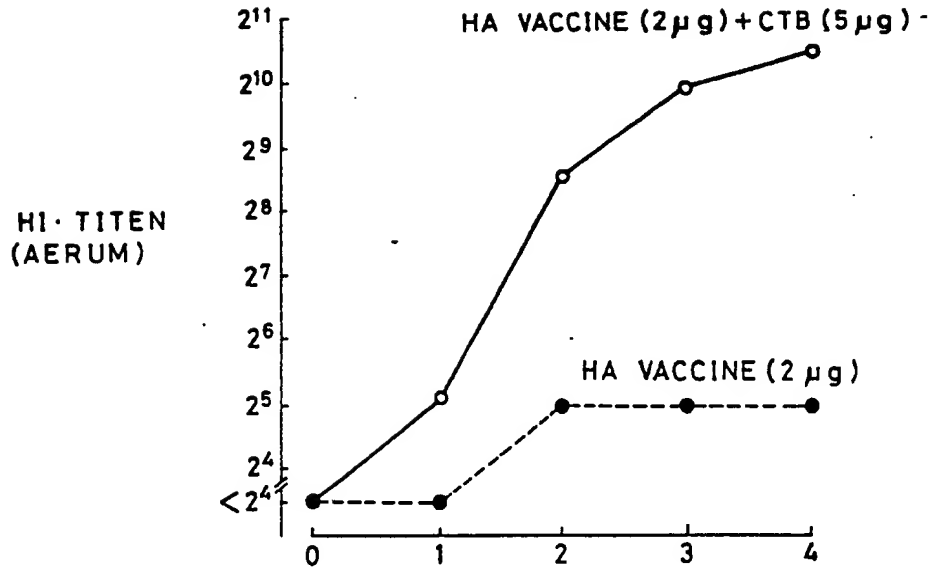

HA VACCINE (2μg)+CTB (5μg)
HA VACCINE (2μg)
HI・TITEN
(AERUM)
$2^{11}$
$2^{10}$
$2^9$
$2^8$
$2^7$
$2^6$
$2^5$
$2^4$
$<2^4$
0
1
2
3
4

FIG. 1B

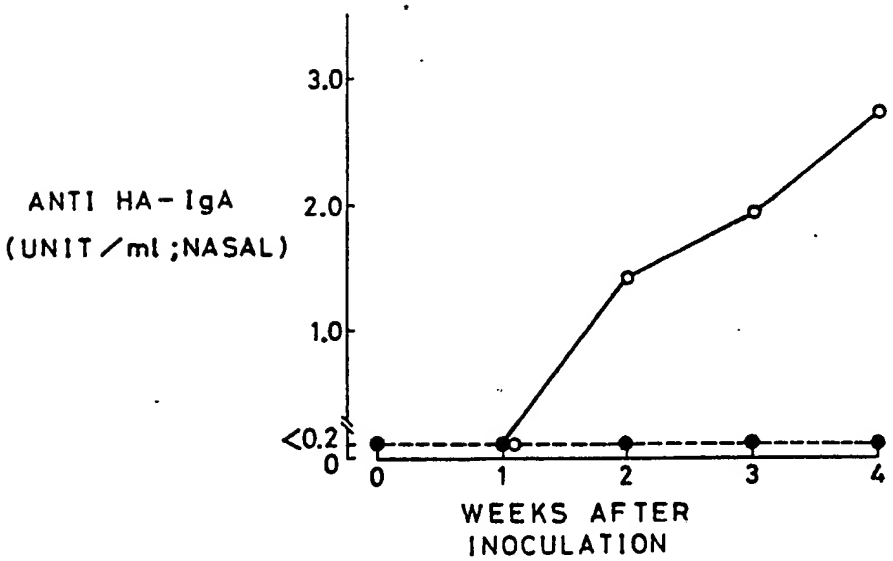

ANTI HA-IgA
(UNIT/ml ;NASAL)
3.0
2.0
1.0
<0.2
0
0
1
2
3
4
WEEKS AFTER
INOCULATION

FIG. 1C

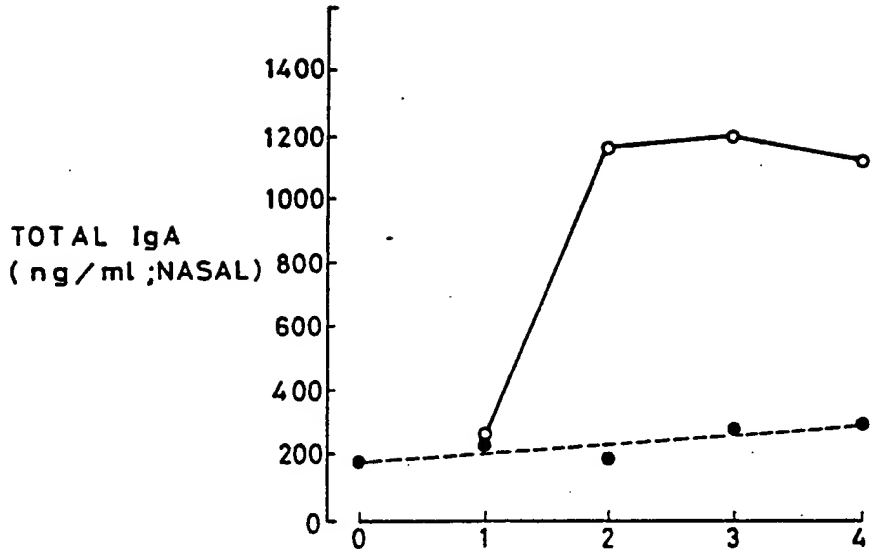

TOTAL IgA
(ng/ml ;NASAL)
1400
1200
1000
800
600
400
200
0
0
1
2
3
4

FIG. 1D

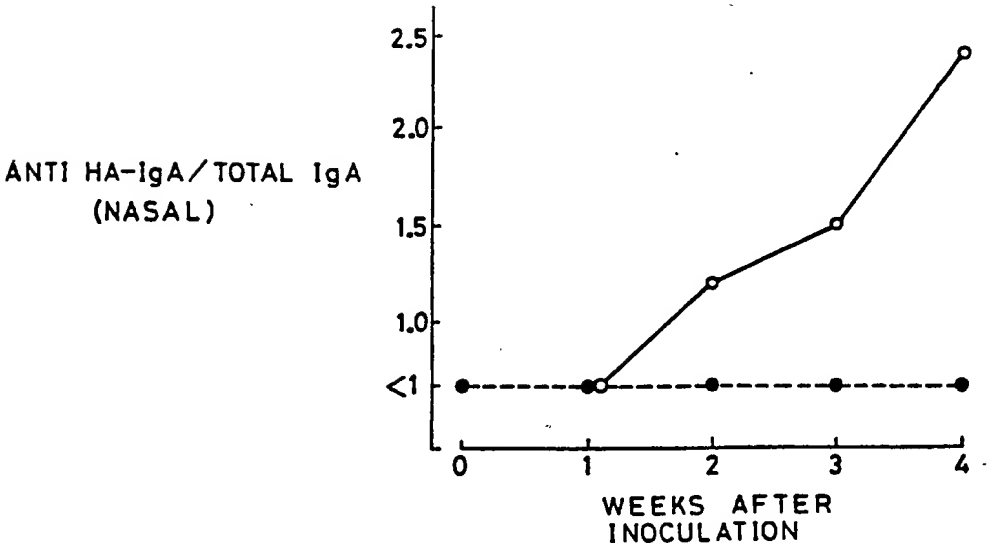
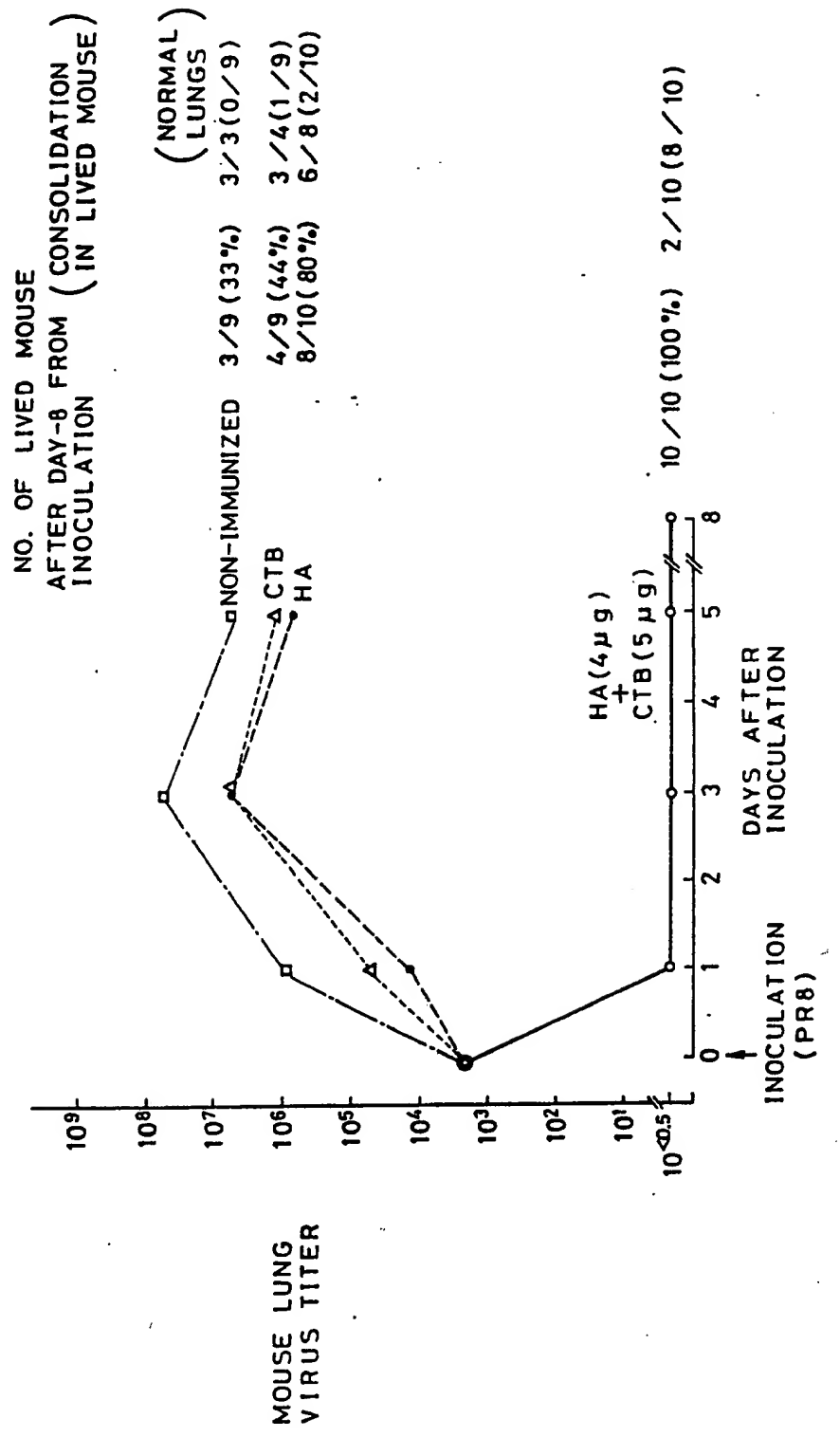

ANTI HA-IgA/TOTAL IgA
(NASAL)
2.5
2.0
1.5
1.0
<1
0
1
2
3
4
WEEKS AFTER
INOCULATION

FIG. 2


NO. OF LIVED MOUSE
AFTER DAY-8 FROM INOCULATION
(CONSOLIDATION IN LIVED MOUSE)
(NORMAL LUNGS)
NON-IMMUNIZED 3/9 (33%) 3/3 (0/9)
CTB 4/9 (44%) 3/4 (1/9)
HA 8/10 (80%) 6/8 (2/10)
HA(4μg) + CTB(5μg) 10/10 (100%) 2/10 (8/10)
MOUSE LUNG VIRUS TITER
$10^9$
$10^8$
$10^7$
$10^6$
$10^5$
$10^4$
$10^3$
$10^2$
$10^1$
$10^{<0.5}$
0 1 2 3 4 5 8
DAYS AFTER INOCULATION
INOCULATION (PR8)

# VACCINE PREPARATION 2217600

This invention relates to a vaccine preparation.

Vaccines are used for protection against various diseases and have provided successful results. However, side effects or insufficient efficacy have sometimes been observed and hence there is a demand for improvement. To reduce side effects it has been proposed to use a more purified vaccine or to administer less vaccine; however these trials have resulted in reduced efficacy of the vaccine.

At present human vaccines are prepared from pathogens or components thereof. Therefore contamination of the pathogen component or of the medium used for culturing the pathogen cannot be avoided. This induces side effects during vaccine inoculation.

The development of an effective vaccine is required with augmented immune potency but without side effects. In order to try to achieve this, various improvements such as decreasing the inoculum size of the vaccine and changing the route of administration have been applied.

We have found that the immune potency of a vaccine can be enhanced by administering the vaccine together with a toxin or a subunit thereof.

The present invention therefore provides a vaccine preparation comprising a vaccine and a toxin or a subunit thereof.

The vaccine preparation of the present invention has enhanced immune potency with decreased level of administration.

The toxin is preferably a bacterial toxin, especially cholera toxin, Staphylococcal α-hemolysin, Staphylococcal δ-hemolysin, Vibrio thermostable direct hemolysin, pertussis toxin or E. coli heat-labile toxin.

The subunit of the toxin is preferably a B subunit or a part thereof.

The vaccine is preferably influenza vaccine, pertussis vaccine, Japanese encephalitis vaccine, a mixed vaccine of pertussis vaccine, diphtheria and tetanus toxoid, hepatitis B vaccine, Rota vaccine, measles vaccine, rubella vaccine, mumps vaccine, a mixed vaccine for measles, rubella and mumps, or mycoplasma vaccine.

The ratio of the vaccine to the toxin or subunit thereof is preferably from 1:0.0001 to 1:10,000 (w/v).

The preparation is preferably an intranasal vaccine preparation. It may be in injectable form, spray form or a form suitable for oral administration.

The vaccine preparation may be used in a method of treatment of the human or animal body by therapy.

Influenza vaccine as a vaccine and cholera toxin (hereinafter designated CT) and cholera toxin B subunit (hereinafter designated CTB) as bacterial toxins are now exemplified to illustrate the present invention.

Cholera toxin is a protein endotoxin produced by Vibrio cholera as a local immunomodulator against vaccine administered intranasally. Cholera toxin is an effective intestinal immunogen and is responsible for diarrhoea in cholera. Moreover, it is known not only as an effective immunogen which induces IgA antibody production, but also as an immunomodulating agent which stimulates the immune responses to simultaneously administered unrelated protein antigens, when CT and the unrelated antigen are administered simultaneously. The effect of CT is defined as an action which increases the intra-cellular cAMP level in intestinal mucosal cells. A mechanism of action of CT, which is composed of an A subunit and a B subunit,

is that CTB binds to $GM_1$ ganglioside in a cell membrane, and A subunit which enters into cell and activates adenylate cyclase. CTB, the nontoxic component of CT, is also shown as a gut mucosal immunogen when administered simultaneously with unrelated proteine antigen. These facts suggest that both CT and CTB can act an respiratory mucosa, as well as on the intestinal mucosa, to stimulate the local immune responses to unrelated coexisting antigens.

We have examined the effectiveness of CT and CTB as an adjuvant on local and serum antibody production for nasal vaccination on mice by using influenza HA vaccine ( hereinafter designates as HA vaccine ). Also the usefulness of CT and CTB as an adjuvant for vaccination effect was examined.

Increased vaccination effect of CTB for nasal vaccination by using HA vaccine on immunoresponse :

Effects of CTB on antibody responses to HA vaccine ( A/Yamagata ) were investigated in mice which received the vaccine together with CTB intranasally. Simultaneously, result of intranasal administration was compared with that of the other inoculation routes.

Four weeks after the nasal vaccination, which was taken by dropping HA vaccine ( 2$\mu$g ) alone or together with CTB ( 5$\mu$g ) intranasally in mice ( or administered intraperitoneally or subcutaneously ), hemagglutinin-

inhibiting ( hereinafter designates as HI ) antibody levels in serum and influenza specific IgA antibody ( hereinafter designates as anti HA-IgA antibody ) and CTB specific IgA antibody ( hereinafter designates as anti CTB-IgA antibody ) in nasal wash were assayed.

As shown in Table 2, control group of mice which received the HA vaccine alone intranasally induced only a low level of serum HI antibodies. Nasal inoculation of CTB together with HA vaccine led to the 64 times higher level of serum HI antibodies. Anti HA-IgA and anti CTB-IgA antibodies in the nasal wash were also observed. Intraperitoneal or subcutaneous inoculation of HA vaccine alone induced a high level of serum HI antibody, and the inoculations of HA vaccine together with CTB induced more enhanced levle as of 4 ~ 8 times higher level of serum HI antibodies. However neither detectable nasal anti HA-IgA nor anti CTB-IgA antibody were induced.

As a result, CTB was an effective adjuvant which stimulate nasal anti HA-IgA antibody production when administered intranasally together with HA vaccine.

Though it is not shown in Table 2, no serum anti HA-IgA was detected.

Effect of CTB on primary antibody response to HA vaccine after intranasal administration :

A progress of antibody production in mice which received intranasally HA vaccine ( A/ Yamagata, 2 μg ) together with CTB ( 5 μg ) was examined.

As shown in Fig. 1, HI antibody production for serum vaccine together with CTB was rapidly increased during 1 to 2 weeks, thereafter increased gradually until 4 weeks period. The total amount of IgA in nasal wash was rapidly increased to maximum level after 1 to 2 weeks of inoculation at approximately 6 times as high as that in control group. The amount of anti HA-IgA therein was gradually increased was gradually increased after 1 week to 4 weeks.

Anti HA antibody in wash nasal began to detectable approximately at 2 weeks after administered both CTB and vaccine.

Effect of CTB on primary and secondary antibody responses to HA vaccine :

Effect of CTB ( 5 μg ) and various amount of HA vaccine ( A/Yamagata ) on anti HA antibody response were investigated.

Primary antibody production was investigated in mice, which received a primary intranasal inoculation of the various does of HA vaccine together with CTB, after 4 weeks of inoculation, then secondary antibody production was

detected further 2 weeks later in mice, which received the second intranasal inoculation of HA vaccine ( 2 $\mu$g ) alone.

As shown in Table 3, a low level of primary antibody production was observed even at 8 $\mu$g of HA vaccine inoculation alone. On the other hand, both nasal anti HI-IgA antibody and serum HI antibody increased with an increase in the intranasal dose of HA vaccine even at 0.03 $\mu$g of inoculation, when CTB was administered concomitantly. Further in mice which received the second intranasal inoculation of HA vaccine alone after the primary inoculation, the level of anti HI and anti HA-IgA antibodies production was enormously elevated, and was independent from the primary HA vaccine dose inoculated with CTB. Mice which received primary inoculation of CTB and HA vaccine showed extremely high levels of anti HI and anti HA-IgA antibodies production were observed, and were indepndent from the amont of primary HA vaccine inoculation. In particular, the level of nasal anti HA-IgA antibody was about 50 - 100 times as high as that after primary inoculation. These results show that the primary inoculated CTB strongly stimulate the antibody production at secondary inoculation without coefficient to an amount of HA vaccine. Namely CTB can effectively produce the immunological memory for the production of nasal IgA antibodies against HA vaccine at relatively low concentration.

Effects of CTB on augmentation of immune response and protection of mice against influenza virus infection :

Immune response against HA vaccine was augmented by concomitantly administered CTB in mice. The effectiveness of CTB as an adjuvant was tested by production experiments using mouse adapted influenza virus, strain PR8. Mice were inoculated intranasally with both of the PR8 HA vaccine ( 1.5 $\mu$g ) and CTB ( 5 $\mu$g ), and 4 weeks later, infected intranasally with PR8 virus. Three days after infection, pulmonary virus titers were determined as an index of protection. As shown in Table 4, complete protection against challenge infection with no detection of virus in lung of mice was provided by the inoculation of vaccine with CTB and the production of high levels of both serum HI antibodies and nasal anti HA-IgA antibodies in mice, and none of mice were infected.

In order to confirm plumonary virus titer after 3 days of viral infection as an indicator of a resistance to infection against viral infection in mice, mice were inoculated with both HA vaccine and CTB, and 4 weeks later, inoculated intranasally with virus strain PR8. After infection, variation of plumonary virus titers were determined.

As illustrated in Fig. 2, mice, which were observed plumonary virus $<10^{0.5}$ after 3 days of infection, showed no detectable virus one day after infection and maintained deminished level with survival thereafter at

least 8 days. On the contrary the lungs of mice in the control groups, which were failed to produce detectable protective antibodies, showed evidence of infection which began to increase one day after infection and reached its maximum after 3 days. Death of mice in the control group were observed after 6 days of infection and in the non-immunized group 6 died in 9 mice were observed after 8 days. Survived 3 mice which showed severe lesion in lung were judged to die within a few days. Similar results as of in non-immunized group were observed in mice inoculated with HA vaccine or CTB alone. It is concluded that plumonary virus titer after 3 days of infection can be the indicator of resistance to infection.

Effect of dose of CTB or CT on the augmentation of immune response and protection of mice against influenza virus infections :

Effects of CT or CTB dose, inoculated intranasally with the PR8 HA vaccine ( 1.5 $\mu$g ), on protection against PR8 virus challange and antibody production were investigated. As shown in Table 5, the slight resistance to infection with a dose of CTB ( 0.05$\mu$g ) was observed, and complete protetion against challange infection was provided by the inoculation of vaccine with 5 $\mu$g of CTB. The degree of resistance to infection seems to be directly proportional to the level of anti HA IgA antibodies in the nasal wash and the level of HI

antibody in the serum on 4 weeks after inoculation of vaccine with CTB.

Complete protection to infecton was observed at the levels of CT more than 0.05 $\mu$g, and under these conditons, the increase of HI antibodies in serum and anti HA-IgA antibodies in the nasal wash was observed according to the increase in the level of CT.

These results suggested that CT is 10 times or more effective than CTB, on a dose basis, for increase HI antibodies production in serum and anti HA-IgA antibodies production in nasal wash necessary for protection to infection.

CT can be used as an effective adjuvant at low dose level as compared with CTB, if there is no side-effect.

It is suggested that HI antibody more than 32 times of antibody titers in serum, and local antibodies more than 2 units of anti HA-IgA antibodies are required for complete protection against PR8 virus infection.

In Table 1, adjuvant activities of various bacterial toxins are illustrated.

TABLE 1

| Toxins | Inoculum size: Toxin (μg/mouse) | Inoculum size: Influenza HA (μg/mouse) | Antibody production HI($2^n$) | No. of mice survived / treated |
|---|---|---|---|---|
| Staphylococcal α-hemolysin | 0.5 | 1.5 | 10.0±1.7 | 5/5 |
| Staphylococcal δ-hemolysin | 5 | 1.5 | <4 | 5/5 |
| | 0.5 | 1.5 | <4 | 5/5 |
| Vibrio thermostable direct hemolysin | 5 | 1.5 | 11.5 | 5/5 |
| | 0.5 | 1.5 | 9.8±1.1 | 5/5 |
| E. coli heat-labile toxin (LT) | 5 | 1.5 | 11.8±0.4 | 5/5 |
| | 0.5 | 1.5 | 10.8±2.2 | 5/5 |
| Pertussis toxin | 5 | 1.5 | 11.6±0.5 | 5/5 |
| | 0.5 | 1.5 | 10.2±1.7 | 5/5 |
| Control | 0 | 1.5 | <4 | 0/5 |
| | | | <4 | 0/5 |

TABLE 2

Augmentation of antiviral antibody response to influenza HA vaccine (A/Yamagata) by CTB

| Group No. | Inoculation Material: HA vaccine (2 μg) | Inoculation Material: CTB (5 μg) | Route | Antibody Responses (4 weeks): Serum HI titer ($2^n$) | Nasal IgA: AntiHA-IgA (units) | Nasal IgA: AntiCTB-IgA (units) |
|---|---|---|---|---|---|---|
| 1 | − | − | − | $< 2^4$ | < 0.2 | < 0.2 |
| 2 | − | + | Intranasal | $< 2^4$ | < 0.2 | 8.3 ± 0.3 |
| 3 | + | − | Intranasal | $2^5$ | < 0.2 | < 0.2 |
| 4 | + | + | Intranasal | $2^{11}$ | 3.3 ± 0.3 | 7.4 ± 1.1 |
| 5 | + | − | Intraperitoneal | $2^8$ | < 0.2 | < 0.2 |
| 6 | + | + | Intraperitoneal | $2^{11}$ | < 0.2 | 0.2 |
| 7 | + | − | subcutaneous | $2^8$ | < 0.2 | < 0.2 |
| 8 | + | + | subcutaneous | $2^{10.5 \pm 0.7}$ | < 0.2 | 0.2 |

TABLE 3

Augmentation of primary and secondary antiviral antibody responses by nasal inoculation of influenza HA vaccine together with CTB

| Group No. | primary Nasal Inoculation HA (μg) | CTB (5 μg) | Antibody Responses after 4 weeks (priary) Serum HI antibody (units) | Nasal Anti IIA-IgA (units) | Antibody Response after 2 weeks Secondary (2 μg IIA vaccine) Serum HI antibody (units) | Nasal Anti IIA-IgA (units) |
|---|---|---|---|---|---|---|
| 1 | - | - | $<2^4$ | $<0.2$ | $2^5$ | $<0.2$ |
| 2 | - | + | $<2^4$ | $<0.2$ | $2^{3.5}\pm^{0.7}$ | $1.2\pm1.2$ |
| 3 | 0.03 | - | $<2^4$ | $<0.2$ | $2^8$ | $2.9\pm1.6$ |
| 4 | 0.5 | - | $<2^4$ | $<0.2$ | $2^{6.5}\pm^{0.7}$ | $2.6\pm0.6$ |
| 5 | 8 | - | $2^5$ | $0.3\pm0.1$ | $2^{8.5}\pm^{0.7}$ | $4.5\pm2.1$ |
| 6 | 0.03 | + | $2^4$ | $0.7\pm0.3$ | $2^{12.5}\pm^{0.7}$ | $60\pm4$ |
| 7 | 0.5 | + | $2^{6.5}\pm^{0.7}$ | $2.5\pm0.4$ | $2^{12.5}\pm^{0.7}$ | $113\pm27$ |
| 8 | 8 | + | $2^{11.5}\pm^{0.7}$ | $4.6\pm0.3$ | $2^{12.5}\pm^{0.7}$ | $78\pm10$ |

TABLE 4

Protection against infection with influenza virus (PR8) inoculated with HA vaccine and CTB

| Group No. | Nasal Inoculation | | Antiviral antibodies after 4 weeks of Inoculation | | Mouse lung titer after 3 days infection | Incidence of infection (%) |
|---|---|---|---|---|---|---|
| | HA vaccine (1.5 μg) | CTB (5 μg) | Serum HI antibody ($2^n$) | Anti IIA-IgA (units) | $EID_{50}$ ($10^n$) | |
| 1 | − | − | $< 2^4$ | < 0.2 | $10^{4.8} \pm^{0.5}$ | 5/5 (100) |
| 2 | − | + | $< 2^4$ | < 0.2 | $10^{4.1} \pm^{0.5}$ | 5/5 (100) |
| 3 | + | − | $< 2^4$ | < 0.2 | $10^{3.7} \pm^{0.5}$ | 5/5 (100) |
| 4 | + | + | $2^{7.3} \pm^{1.3}$ | 4.6±0.7 | $10^{<0.5}$ | 0/5 (0) |

TABLE 5

Effect of CTB or CT dose on protection against infection

| Group No. | Nasal Inoculation: HA vaccine (1.5 μg) | Nasal Inoculation: CTB (CT) (μg) | Antibody Responses (4 weeks after Inoculation): Serum HI antibody titer ($2^n$) | Nasal: Anti HA-IgA (units) | Nasal: Anti CTB-IgA (units) | Mouse lung after 3 days of infection ($ED_{50}$) ($10^n$) |
|---|---|---|---|---|---|---|
| 1 | − | − | $<2^4$ | <0.2 | <0.2 | $10^{6.8}$ |
| 2 | − | CTB (5) | $<2^4$ | <0.2 | 6.2±1.3 | $10^{7.1}$ |
| 3 | + | − | $<2^4$ | <0.2 | <0.2 | $10^{3.7}$ |
| 4 | + | CTB (0.05) | $<2^4$ | 0.5 | <0.2 | $10^{3.9}$ |
| 5 | + | CTB (0.5) | $2^{4.5}\pm^{0.7}$ | 1.2±0.9 | 1.5±1.0 | $10^{2.5}$ |
| 6 | + | CTB (5) | $2^{6.5}\pm^{0.7}$ | 4.6±0.7 | 3.7±0.6 | $10^{<0.5}$ |
| 7 | + | CT (0.05) | $2^5$ | 2.0±0.8 | <0.2 | $10^{<0.5}$ |
| 8 | + | CT (0.5) | $2^7$ | 3.2±0.6 | 0.6±0.6 | $10^{<0.5}$ |
| 9 | + | CT (5) | $2^{6.5}\pm^{0.7}$ | 3.1±0.1 | 2.7±0.1 | $10^{<0.5}$ |

As hereinabove illustrated following facts have been found.

(1) Administration of influenza HA vaccine together with CT or CTB augments the immune production.

(2) Augmentation of serum HI antibodies production and local intranasal anti HA-IgA production are augmented by intranasal inoculation of HA vaccine with CTB. Almost no local intranasal HA-IgA antibodies production are not observed when inoculated intraperitoneally and subcutaneously, but the high level of antibody production in serum found.

As a result CTB reveals augmentation activity of antibody production at any inoculation routes.

This facts means mixed administration of CT, CTB, Staphylococcal $\alpha$-hemolysin, Staphylococcal $\delta$-hemolysin, Vibrio thermostable direct hemolysin, pertussis toxin or E. coli heat-labile toxin with pertussis vaccine, diphtheria and tetanus toxid combined with pertussis vaccine, hepatitis B vaccine, Japanese encephalitis vaccine, measles vaccine, rubella vaccine, mumps vaccine, measles, rubella and mumps mixied vaccine, rota vacine or mycoplasma vaccine reveals high level of titer as compared with that of single dose of vaccine.

In other word, inoculum size of vaccine can be reduced by administering together with toxin and subunit thereof.

(3) The level of local nasal anti HA-IgA antigen is

increased on 2 to 4 weeks by intranasally inoculating vaccine with CTB.

(4) Antibody production on 4 weeks after intranal inoculation of vaccine with CTB is propotional to a dose level of inoculated vaccine.

(5) In mice which received the second intranasal inoculation of the vaccine alone 4 weeks after the primary inoculation of the vaccine with CTB, the level of secondary antibody production 2 weeks after the second inoculation was enormously elevated, and was independent of the primary vaccine dose inoculated with CTB. Therefore CTB can effectively produce the immunological memory.

(6) Complete protection against influenza virus infection was provided by the intranasal inoculation of vaccine with CTB in mice, in which the production of high levels of both serum antibody titers and nasal anti HA-IgA antibodies were observed.

In the present experimental condition, in mice which maintain serum HI antibodies more than 32 times of antibody titers and local nasal antibodies more than 2 units, complete protection against PR8 virus infection.

(7) Inoculum size of CTB necessary for induction of antibody production for complete protection to infection, intranasally inoculated with vaccine is an order of $\mu$g. CT can induce antibody production which requires for complete protection to infection at level below 1/10 as of CTB.

Toxins or subunit thereof used in the present invention, such as CT or CTB, can be prepared by the known procedures, and is also commercially available. CT is toxic when administered with large amount but is non toxic when intranasally or intraperitoneally inoculated CTB is less toxic than CT and is no problem in an intranasal administration. Examples of further toxins are Staphylococcal $\alpha$-hemolysin, Staphylococcal $\delta$-hemolysin, Vibrio thermostable direct hemolysin, pertussis toxin and E. coli heat-labile toxin.

Examples of vaccine are influenza vaccine, pertussis vaccine, diphtheria and tetanus toxoid combined with pertussis vaccine, hepatitis B vaccine, Japanese encephalitis vaccine, measles vaccine, rubella vaccine, mumps vaccine, mixed vaccine of measles, rubella and mumps, rota vaccine and mycoplasma vaccine, and others. These can be produced by known common process. Production and process thereof are illustrated hreinbelow.

Influenza vaccine : a vaccine comprising whole or part of hemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP) and matrix protein (M) which are obtainable by purifying virus, which is grown in embryonated eggs, with ether and detergent, or by genetic engineering techniques or chemical synthesis.

Pertussis vaccine : a vaccine comprising whole or part of pertussis toxin (PT), hemagglutinin (FHA) and

K-agglutin in which are obtained from avirulent toxin with formalin which is extracted by salting-out or ultracentrifugation from culture broth or bacterial cells of Bordetella pertussis, or by genetic engineering techniques or chemical synthesis.

Diphtheria and tetanus toxoid combined with pertussis vaccine : a vaccine mixed with pertussis vaccine, diphtheria and tetanus toxoid.

Japanese encephalitis vaccine : a vaccine comprising whole or part of antigenenic protein which is obtained by culturing virus intracerebrally in mice and purifying the virus particles with centrifugation or ethyl alcohol and inactivaing the same, or by genetic engineering thechnique or chemicalsynthesis.

Hepatitis B vaccine : a vaccine comprising whole or part of antigen protein which is obtained by isolating and purifying the HBs antigen with salting-out or ultracentrifugation obtained from hepatitis B carrying blood, or by genetic engineering or chemical synthesis.

Measles vaccine : a vaccine comprising whole or part of virus grown in cultured chick embryo cells or embryonated egg, or protective antigen obtained by genetic engineering technique or chemical synthesis.

Rubella vaccine : a vaccine comprising whole or part of virus grown in cultured chick embryo cells or embryonated egg, or protective antigen obtained by genetic engineering

technique or chemical synthesis.

Mumps vaccine : a vaccine comprising whole or part of virus grown in cultured rabbit cells or embryonated egg, or protective antigen obtained by genetic engineering technique or chemical synthesis.

Mixed vaccine of measles, rubella and mumps : a vaccine comprising mixing measles, rubella and mumps vaccines.

Rota vaccine : a vaccine comprising whole or part of virus grown in cultured MA 104 cells, or isolated from patients' feces, or protective antigen obtained by genetic engineering technique or chemical synthesis.

Mycoplasma vaccine : a vaccine comprising whole or part of mycoplasma cells grown in liquid culture medium for mycoplasma, or protective antigen obtained by genetic engineering technique or chemical synthesis.

The above illustrated vaccines are provide with liquid or powdered form.

Liquid form of vaccine is preferable for intranasal administration such as intranasal spray, dripping or applying or injection together with toxin or subunit thereof. Powder spraying intranasally can also be applicable. Amount of dose may be 5 $\mu\ell$ ~ 50 $\mu\ell$ intranasally in mice and is preferable at 0.1 ~ 0.5 m$\ell$ for intranasal or injection in man. These amount of administration can also be modified accordingly.

Mixed ratio vaccine and toxin or its subunit is 1 : 0.001 ~ 1 : 10,000 (w/v %) and depends on an amount of dose in man.

Vaccine of the present invention can be prepared by mixing the above illustrated vaccine with toxin or subunit thereof at desired ratio. Preparation should be done strictly with aseptically, and each material should also be aspectic. Pyrogen or allergen should naturally be removed as completely as possible.

Vaccine preparation of the present invention can be used by preparing vaccine per se and toxin or subunit thereof separately and mixing before inoculation or by inoculating each separately.

Following referential example illustrates the vaccine of the present invention comprising influenza vacine and CT or CTB and effect thereof.

Referential example

Animal : Female Balb/c mice, 6 to 8 weeks old.

HA vaccine : HA vaccines were prepared from influenza virus by treatment with ether to remove lipid composition. HA component approximately 30 % is contained in the HA vaccine. Inoculum size shown in Table 2 - 5 are expressed as an amount of HA.

CT and CTB : Cholera toxin (CT) and its B subunit (CTB) were purchased from Sigma Chemical Co., U.S.A. The CTB preparation used in the present experiments did not reveal any detectable contamination with A subunit as determined by SDS-polyacrylamide gel electrophoresis.

Imuunization :

A vaccine preparation was prepared by diluting HA vaccine or adjuvant with phosphate buffered saline (PBS). Mice were an aesthetized by an intraperitoneal injection of amobarbital sodium and then immunized by an intranasal dripping inoculation of 20 $\mu\ell$ of the vaccine preparation. Alternatively they were immunized by a subcutaneous or an intraperitoneal injection of 100 $\mu\ell$ of the vaccine.

Serum and nasal washed specimens :

Serum specimens were collected from mice by drawing whole blood from their heart. Nasal wash specimens were obtained by washing the nasal cavities, each two times with a total of 1 m$\ell$ PBS.

Hemagglutination inhibition (HI) test :

Serum for HI titer assay was prepared after removal of non-specific inhibitors by receptor destroying enzyme (RED). Serum was diluted with 2 fold dilution on U-type microtiter plate and mixed with virus of 16 HA units, which were allowed to stand for 30 minutes at room temperature. Then chick red cells were added thereto for assaying.

Results were determined after standing at room

temperature for 1 hours.

Assay of anti HA-IgA, anti CTB-IgA and total IgA :

Antiviral IgA, anti CTB antibodies and total IgA in nasal wash and serum measured by ELISA. The wells of 96-well EIA plate (half-area, Costar, Cambridge, MA) were coated with either 50 $\mu\ell$ of HA vaccine (5 $\mu$g/ m$\ell$) or CTB (5$\mu$g/ m$\ell$). The plate was incubated for 2 hours at room temperature and then washed three times with PBS-Tween. The PBS solution (100 $\mu\ell$) containing 1 % bovine serum albumin (BSA) and 0.1 % $NaN_3$, was placed in the wells for coating, and incubated overnight at 4 ℃. After washing with PBS-Tween, each sample of either serum or nasal wash specimen was added to the wells. The plates were incubated for 2 hours at room temperature and washed with PBS-Tween.

Alkaline-phosphatase-coupled goat anti-mouse IgA ( $\alpha$-chain specific, 1 : 1000, Zymed Laboratories, Inc. U.S.A., 50$\mu\ell$) was added to each well. The palte was incubated at room temperature for 1 hour and then washed with PBS-Tween. Finally p-nitrophenylphosphate (1mg/ m$\ell$, Sigma Co.) in 10 % diethanolamine buffer at pH 9.8 (100$\mu\ell$) was added to each well.

After 20 - 30 minutes, the absorbance of the plates was read at 410 nm ($OD_{410}$) in a SJeia Auto Reader (model ER-8000, Sanko Junyaku Co., Tokyo) Standard curve was prepared for each plate. A standard regression curve, which is transformed by a logit-log equation, was constructed for each

assay and unknowns were interpolated using a programm on the SJeia Auto Reader.

A standard anti HA-IgA measurement solution is defined as an 8-unit standard for antiviral IgA antibodies, which is nasal specimens from mice which received 5 times repeated nasal inoculations of the HA vaccine at 2-week intervals. A standard anti CTB-IgA measurement solution is defined as an 8-unit standard for antiviral IgA antibodies, which is nasal specimens from mice 4 weeks after the nasal inoculations of CTB ( 5 $\mu$g ).

Total IgA was determined by the same was as illustrated hereinabove except that the goat anti-mouse IgA (50 $\mu\ell$, 1 $\mu$g/ m$\ell$) was added into each well. Mouse purified IgA myeloma (Miles Labs. U.S.A.) was used as a standard solution for total IgA measurement.

Infection with PR8 virus in mice :

Mice were an aesthetized and then infected by intranasal dropping administration of 20 $\mu\ell$ virus suspension containing 0.01 % BSA into left nasal cavity. Virus suspension was prepared as a 1/5000 ~ 10000 suspension of original virus pool with $10^{8.5}$ $EID_{50}$ which was prepared by inoculating 148 transfers in ferrets, 596 transfers in mice and 72 transfers in mice and 72 transfers in embryonated eggs. In this infectious condition, more than 90 % of non-immunized were died within 14 days or formed consolidation in lungs.

Virus titrations in lung :

Three days after infection, lungs of mice were removed, homogenized to give a 10 % suspension in PBS and then centrifuged at 2500 rpm. Serial dilutions ( 1:10 ) of the supernatant of individual lung homogenates were prepared and each dilution was injected into 5 embryonated eggs. The lung virus titer of each mouse was determined by the hemmaglutinating capacity of the allantoic fluid and expressed by the lowest dilution of lung homogenate with $EDI_{50}$ that was infection in the eggs.

The lung virus titer was expressed by mean $\pm$ SD. In some experiments, lungs of 5 mice in a group were combined to prepare lung suspension.

Incidence infection :

Incidence of infection values were represented by the number of infected mice, in which the virus was present at $>10^1$ in the lung homogenate (10 %), per 5 tested mice.

Statistics :

Probability values were calculated by student's t-test.

Following examples illustrate the present invention but are not construed as limiting.

Example 1

Influenza HA vaccine-CTB (Intranasal preparation):

Influenza HA vaccine (1 mg HA/ m ℓ) and CTB, which was dissolved in PBS and aseptically filtrated, were mixed to prepare the influenza HA vaccine-CTB, intranasal splution (20 μ ℓ) containing influenza HA vaccine (1.5～ 2 μg) and CTB (3.5～ 250 μg) together with adding preservative and stabilizer, filled into bottles. The vaccine preparation is stored in dark-cool place below 10 ℃. Effect of the vaccine preparation is illustrated hereinbefore.

Example 2

Influenza HA vaccine-CTB (Injection):

Influenza HA vaccine (1 mg HA/ m ℓ) and CTB, which was dissolved in PBS and asseptically filtrated, were mixed to prepare the influenza HA vaccine-CTB, injectable solution (0.5 m ℓ) containing influenza HA vaccine (1.5～ 2 μg) and CTB (2.5～ 250 μg) together with adding preservative and stabilizer, filled into vials. The vaccine preparation is stored in dark-cool place below 10℃. Effect of the vaccine preparation is illustrated hereinbefore.

Example 3

Hepatitis B vaccine-CTB (Injection):

Hepatitis B vaccine-CTB for injection was prepared by mixing hepatitis B vaccine and CTB, which was dissolved in PBS and aseptically filtrated, to prepare a mixture containing HBs antigen (40 $\mu$g protein) and CTB (2.5 $\sim$ 250 $\mu$g) in 20 $\mu\ell$ solution, together with adding preservative and stabilizer, filled into vials. The vaccine preparation is stored in dark-cool place below 10 ℃.

The thus prepared hepatitis B vaccine preparation and CT were inoculated in mice and sereum titer after 3 weeks of inoculaton was measured.

As shown in the table, mice received hepatitis B vaccine showed passive hemagglutination (PHA) titer of $2^{5.6}$ units and mice received additional CT showed PHA titer of $2^{6.6}$ units, and hence antibodies production were augmented.

Table : Augmentation of antibody production by adding CT with hepatitis B vaccine

| | Antibody titer PHA |
|---|---|
| CT | $2^{6.6}$ |
| no addition | $2^{5.6}$ |

Antibody titer was measured by the passive hemagglutination test. Antibody titers are expressed by average of 5 mice.

Example 4

Pertussis vaccine-CTB (Intranasal) :

Pertussis vaccine and CTB, dissolved in PBS and filtrated aseptically, were mixed to prepare the pertussis vaccine-CTB intranasal by preparing a mixture of pertussis vaccine (14 $\mu$g protein nitrogen) and CTB (25 ~ 250 $\mu$g) in 20 $\mu\ell$, together with preservative and stabilizer and packed in a bottle. The product is stored in a dark-cool place below 10℃.

CTB or CT was added to the pertussis vaccine 20 $\mu\ell$ (13 $\mu$g protein nitrogen), and inoculated to mice intranasally. After 4 weeks, the same inoculum size of vaccine was administered intranasally into mice, and antibodies titer were determined.

As shown in the table, mice received pertussis vaccine alone showed anti PT antibody of <4.1 units, and pertussis vaccine together with CTB or CT showed anti PT antibody of 140.3 units or 232.5 units, respectively. Anti FHA antibodies of mice inoculated with vaccine alone, adding CTB or CT were <2.6 units, <32.0 units and 43.9 units, respectively. Antibody production was augmented in mice when administered vaccine and CTB or CT.

Table : Augmentation of antibody production by pertussis vaccine adding with CT or CTB

| | Anti titer | |
|---|---|---|
| | antiPT | antiFHA |
| CT | 232.5 | 43.9 |
| CTB | 140.3 | 32.0 |
| no addition | < 4.1 | < 2.6 |

Titers : average of 5 mice

Titers : ELISA international unit

Example 5

Diphteria and tetanus toxoid combined with pertussis vaccine-CTB (Intranassaly):

Diphtheria and tetanus toxoid combined with pertussis vaccine-CTB intranasal preparation was prepared by mixing a diphtheria and tetanus toxoid combined with pertussis vacine (hereinafter designates as combined vaccine) with CTB dissolved in PBS and filtrated aseptically, preparing a mixture (20 μℓ) of the combined vaccine (50 μg protein nitrogen) and CTB (2.5～ 250μg), added preservative and stabilizer, and filled

in a bottle.

The preparation is stored in a dark-cool place below 10℃.

CTB was added to the combined vaccine, inoculated to mice intranasally and after 4 weeks further administered the same inoculum size, then measured the antibody titers.

As shown in the table, mice received the combined vaccine alone showed anti pertussis toxin (PT), antibody <2.0 units, anti diphtheria (DT) antibody <1.5 unit and anti tetanus toxoid (TT) <1.5 unit. Antibodies production were augmented by adding with CTB to 150.0, 110.5 and 120.0 units, respectively as above.

Table : Augmentation of antibody production by adding CTB in the combined vaccine

| Antigen : inoculum size | CTB | Antibody production ELISA-titer |
|---|---|---|
| Pertussis vaccine 14μg | 5μg | 150.0 |
| Diphtheria toxoid 16μg | | 110.5 |
| Tetanus toxoid 15μg | | 120.0 |
| Pertussis vaccine 14μg | 0μg | < 2.0 |
| Diphtheria toxoid 16μg | | < 1.5 |
| Tetanus toxoid 15μg | | < 1.5 |

Antibody titer : mean of 5 mice

Anti titer : ELISA I.U.

Example 6

Japanese encephalitis vaccine-CTB (injection):

Japanese encephalitis vaccine-CTB (injection) was prepared by mixing Japanese encephalitis virus vaccine with CTB dissolved in PBS and filtrated aseptically, preparing mixture (1 mℓ) of inactivated virus particles corresponding to Japanese encephalitis $10^{7.0}$ PFU and CTB (10.0~ 0 μg), added preservative and stabilizer, and filled in a vial.

The preparation is stored in a dark-cool place below 10℃.

Japanese encephalitis vaccine together with or without CTB ot CT was inoculated in mice 2 times with 1 week interval and serum antibody titer was measured.

Neutralizing antibody titers of Japanese encephlitis virus with or without CTB or CT are $10^{1.88}$, $>10^{2.58}$ and $>10^{3.20}$, respectively, and the antibody production was augmented by adding CTB or CT in vaccine.

Table : Augmentation of antibody production by adding CT or CTB in Japanese encephalitis vaccine

| Concentration | | Neutralizing titer $10^n$ |
|---|---|---|
| CT | 0.05μg/Mouse | 3.39 |
| | 0.5 μg | 3.20 |
| | 5.0 μg | 3.52 |
| CTB | 0.05μg/Mouse | 2.58 |
| | 0.5 μg | 2.70 |
| | 5.0 μg | 3.39 |
| no add. | | 1.88 |

Antibody titers of pooled serum of 10 mice

Example 7

Measles vaccine-CTB (intranasal) :

Measles vaccine-CTB intranasal preparation was prepared by mixing measles vaccine with CTB dissolved in PBS and filtrated aseptically, preparing a mixture (20 $\mu\ell$) of virus particles corresponding to measles vaccine (20 $\mu\ell$) and CTB (5 $\mu$g), added preservative and stabilizer, and filled in a bottle.

The preparation is stored in a dark-cool place below 10℃.

Measles vaccine with or without CTB was inoculated 2 times in mice with 3 weeks interval, and measured the serum antibody production.

ElISA antibody titer administered measles vaccine alone was 0.144, and that of CTB added vaccine was >0.209. Augmentation of antibody production was observed when administered vaccine with CTB.

Table : Augmentation of antibody production by adding CTB in measles vaccine

| Inoculum size | | Antibody production ELISA-titer |
|---|---|---|
| Antigen | CTB | |
| Measles vaccine 20 μg | 5 μg | 0.21 |
| Control 20 μg | 5 μg | 0.144 |

Example 8

Rubella vaccine-CTB (intranasal):

Rubella vaccine-CTB intranasal preparation was prepared by mixing rubella vaccine with CTB dissolved in PBS and filtrated aseptically, preparing a mixture (20 μℓ) of virus particles corresponding to rubella vaccine (20 μℓ) and CTB (5 μg), added preservative and stabilizer, and filled in a bottle.

The preparation is stored in a dark-cool place below 10℃.

Rubella vaccine with or without CTB was inoculated 2 times in mice with 3 weeks interval, and measured the serum

antibody production.

ELISA antibody titer administered rubella vaccine alone was 0.095, and that of CTB added vaccine was >0.920. Augmentation of antibody production was observed when administered vaccine with CTB.

Table : Augmentation of antibody production by adding CTB in rubella vaccine

| Inoculum size | | Antibody production ELISA-titer |
|---|---|---|
| Antigen | CTB | |
| Rubella vccine 20 μg | 5 μg | 0.920 |
| Control 20 μg | 0 μg | 0.095 |

Example 9

Mumps vaccine-CTB (intranasal):

Mumps vaccine-CTB intranasal preparation was prepared by mixing mumps vaccine with CTB dissolved in PBS and filtered aseptically, preparing a mixture (20 μℓ) of virus particles corresponding to mumps vaccine (20 μℓ) and

CTB (5μg), added preservative and stabilizer, and filled in a bottle.

The preparation is stored in a dark-cool place below 10℃.

Mumps vaccine with or without CTB was inoculated 2 times in mice with 3 weeks interval, and measured the serum antibody production.

ELISA antibody titer administered mumps vaccine alone was 0.028, and that of CTB added vaccine was >0.045. Augmentation of antibody production was observed when administered vaccine with CTB.

Table : Augmentation of antibody production by adding CTB in mumps vaccine

| Inoculum size | | Antibody production |
|---|---|---|
| Antigen | CTB | ELISA-titer |
| Mumps vaccine 20μg | 5μg | 0.05 |
| Control 20μg | 0μg | 0.028 |

Example 10

Mixed vaccine of measles, rubella and mumps-CTB (intranasal) :

Mixed vaccine of measles, rubella and mumps-CTB intranasal preparation was prepared by mixing the mixed vaccine with CTB dissolved in PBS and filtrated aseptically, preparing a mixture (20 $\mu\ell$) of virus particles corresponding to measles vaccine (7 $\mu$g), rubella vaccine (1 $\mu$g) and mumps vaccine (7 $\mu$g), and CTB (5 $\mu$g), added preservative and stabilizer, and filled in a bottle.

The preparation is stored in a dark-cool place below 10℃.

The vaccine with or without CTB was inoculated 2 times in mice with 3 weeks interval, and measured the serum antibody production.

ELISA antibody titer administered the vaccine alone was 0.14, 0.09 and 0.15, respectively for measles, rubella and mumps, and that of CTB added vaccine was 0.29, 0.30 and 0.24, respectively. Augmentation of antibody production was observed when administered vaccine with CTB.

Table : Augmentation of antibody production by addition of CTB in the nixed vaccine of measles, rubella and mumps

| Inoculum size | | Antigen production ELISA-titer |
|---|---|---|
| Antigen | CTB | |
| Measles vaccine 7 μg<br>Rubella vaccine 7 μg<br>Mumps vaccine 7 μg | 5 μg | Measles 0.29<br>Rubella 0.30<br>Mumps 0.24 |
| Control | | Measles 0.14<br>Rubella 0.09<br>Mumps 0.15 |

Example 11

Rota vaccine-CTB (oral and intranasal) :

Rota vaccine-CTB oral and intranasal preparation was prepared by mixing rota vaccine with CTB dissolved in PBS and filtrated aseptically, preparing a mixture (20 $\mu\ell$) of virus particles corresponding to rota vaccine (3.3 $\mu\ell$) and CTB (5 $\mu$g), added stabilizer, and filled in a bottle.

The preparation is stored in a dark-cool place below 10 ℃.

Rota vaccine with or without CTB was inoculated 2 times in mice with 3 weeks interval, and measured the serum antibody production.

ELISA antibody titer of administered vaccine alone was 0.089 for intranasal administration, and that of CTB added vaccine was 0.281, and 0.018 for oral administration and 0.277 for CTB added vaccine with oral administration.

Augmentation of antibody production was observed when administered vaccine with CTB.

Table : Augmentation of antibody production by addition of CTB in rota vaccine

| Inoculum size | | | | | Antibody Production |
|---|---|---|---|---|---|
| | | Antigen | | CTB | |
| Rota vaccine | Intranasal | Vaccine | 3.3 μg | 5 μg | 0.281 |
| | | Control | 3.3 μg | 0 μg | 0.089 |
| | Oral | Vaccine | 3.3 μg | 5 μg | 0.227 |
| | | Control | 3.3 μg | 0 μg | 0.018 |

Example 12

Mycoplasma vaccine-CTB (injection) :

Mycoplasma vaccine-CTB injectable preparation was prepared by mixing mycoplasma vaccine with CTB dissolved in PBS and filtrated aseptically, preparing a mixture (1 m ℓ) of mycoplasma corresponding to vaccine ($2.0 \times 10^{10}$ CFU) (colony

forming unit) and CTB (10 μg), added stabilizer, and filled in a vial.

The preparation is stored in a dark-cool place below 10℃.

The vaccine with or without CTB was inoculated 3 times in chicken with 2 weeks interval, and observed the number of lesions after challenge of mycoplasma infection.

The administration of CTB added vaccine showed marked protective effect as compared with that of the vaccine alone.

Table : Decrease of lesion after challenge of mycoplasma infection administered mycoplasma vaccine with CTB

| Inoculum size | | Lesion | |
|---|---|---|---|
| Antigen | CTB | | |
| Total mycoplasma cells*** 1.0×10¹⁰CFU | 5 μg | * 3/10 | ** 125 |
| Ultrasonication 1.0×10¹⁰CFU | 5 μg | 3/11 | 129 |
| Control 1.0×10¹⁰CFU | 0 μg | 10/10 | 277 |

* : No. of animals showing lesions/No. of animals tested

* * : mean value of lesions

* * * : CFU

Example 13

Pertussis vaccine-LTB (intransal) :

Pertussis vaccine-LTB (intranasal) was prepared by mixing pertussis vaccine with LTB dissolved in PBS and filtrated aseptically, preparing a mixture (20 $\mu\ell$) of pertussis vaccine (14 $\mu$g protein nitrogen) and LTB (2.5~ 250 $\mu$g), adding preservative and stabilizer, and packing in a bottle.

The preparation is stored in a dark-cool place below 10℃.

LTB or LT was added to the pertussis vaccine 20 $\mu\ell$ (13 $\mu$g protein nitrogen), and inoculated to mice intranasally. After 4 weeks, the same inoculum size of vaccine was administered intranasally into mice, and antibodies titer were determined.

As shown in the table, mice received pertussis vaccine alone showed anti PT antibody of <4.2 units, and pertussis vaccine together with CTB or CT showed anti PT antibody of 150.3 units or 230.5 units, respectively. Anti FHA antibodies of mice inoculated with vaccine alone, adding CTB or CT were <2.3 units, <30.0 units and 40.5 units, respectively. Antibody production was augmented in

mice when administered vaccine and LTB or LT.

Table : Augmentation of antibody production by pertussis vaccine adding with LT or LTB :

| | Antibody titer | |
|---|---|---|
| | AntiPT | AntiFHA |
| LT | 230.5 | 40.5 |
| LTB | 150.3 | 30.0 |
| no add | < 4.2 | < 2.3 |

Antibody titer : mean of 5 mice

Antibody titer : ELISA I.U.

Example 14

Diphtheria and tetanus toxoid combined with pertussis vaccine-LTB (intranasal) :

Diphtheria and tetanus toxoid combined with pertussis vaccine-LTB intranasal preparation was prepared by mixing a diphtheria and tetanus toxoid combined with pertussis vaccine (hereinafter designates as combined vaccine) with LTB dissolved in PBS and filtered aseptically, preparing a mixture (20 $\mu\ell$) of the combined vaccine (50 $\mu$g protein nitrogen)

and LTB (2.5~ 250 $\mu$g), added preservative and stabilizer, and filled in a bottle.

The preparation is stored in a dark-cool place below 10℃.

LTB was added to the combined vaccine, inoculated to mice intranasally and after 4 weeks further administered with the same inoculum size, then measured the antibody titers.

As shown in the table, mice received the combined vaccine alone showed anti pertussis toxin (PT) antibody of <1.8 I.U., anti diphtheria (DT) antibody <1.4 unit and anti tetanus toxoid (TT) 1.2 unit. Antibodies production were augmented by 0.3 adding with LTB to 140.0, 80.5 and 100.5 I.U., respectively.

Table : Augmentation of antibody production by adding LTB in the combined vaccine

| Antigen : Inoculum size | L T B | Antibody production ELISA-titer |
|---|---|---|
| pertussis vaccine 14 μg | | 140. 0 |
| Diphtheria toxoid 16 μg | 5 μg | 80. 5 |
| Tetanus toxoid 15 μg | | 100. 2 |
| Pertussis vaccine 14 μg | | < 1. 8 |
| Diphtheria toxoid 16 μg | 0 μg | < 1. 4 |
| Tetanus toxoid 15 μg | | < 1. 2 |

Antibody titer : mean of 5 mice

Antibody titer : ELISA I. U.

Example 15

Rubella vaccine-LTB (intransal) :

Rubella vaccine-LTB intranasal preparation was

prepared by mixing rubella vaccine with LTB dissolved in PBS and filtrated aseptically, preparing a mixture (20 μℓ) of virus particles corresponding to rubella vaccine (3 μg) and LTB (5 μg), adding stabilizer, and filling in a bottle.

The preparation is stored in a dark-cool place below 10℃.

Rubella vaccine with or without LTB was inoculated 2 times in mice with 3 weeks interval, and measured the serum antibody production.

ELISA antibody titer administered rubella vaccine alone was 0.133, and that of LTB added vaccine was >0.854. Augmentation of antibody production was observed when administered vaccine with LTB.

Table : Augmentation of antibody production by an addition of LTB in rubella vaccine

| Inoculum size | | Antibody production |
|---|---|---|
| Antigen | LTB | ELISA-titer |
| Rubella vaccine 3 μg | 5 μg | 0.854 |
| Control 3 μg | 0 μg | 0.133 |

Example 16

Measles vaccine-LTB (intransal) :

Measles vaccine-LTB intranasal preparation was prepared by mixing measles vaccine with LTB dissolved in PBS and filtrated aseptically, preparing a mixture (20 $\mu\ell$) of virus particles corresponding to measles vaccine (20 $\mu$g) and LTB (5 $\mu$g), added stabilizer, and filled in a bottle.

The preparation is stored in a dark-cool place below 10℃.

Measles vaccine with or without LTB was inoculated 2 times in mice with 3 weeks interval, and measured the serum antibody production.

ELISA antibody titer administered measles vaccine alone was 0.182, and that of LTB added vaccine was >0.332. Augmentation of antibody production was observed when administered vaccine with LTB.

Table : Augmentation of antibody production by an addition of LTB in measles vaccine

| Inoculum size | | Antibody production ELISA-titer |
|---|---|---|
| Antigen | LTB | |
| Measles vaccine 20 μg | 5 μg | 0.332 |
| Control 20 μg | 0 μg | 0.182 |

Example 17

Mumps vaccine-LTB (intranasal):

Mumps vaccine-LTB intranasal preparation was prepared by mixing mumps vaccine with LTB dissolved in PBS and filtered aseptically, preparing a mixture (20 μℓ) of virus particles corresponding to mumps vaccine (20 μg) and LTB (5 μg), added stabilizer, and filled in a bottle.

The preparation is stored in a dark-cool place below 10℃.

Mumps vaccine with or without LTB was inoculated 2 times in mice with 3 weeks interval, and measured the serum antibody production.

ELISA antibody titer administered measles vaccine

alone was 0.023, and that of LTB added vaccine was >0.074. Augmentation of antibody production was observed when administered vaccine with LTB.

Table : Augmentation of antibody production by an addition of LTB in mumps vaccine

| Inoculum size | | Antibody production |
|---|---|---|
| Antigen | LTB | ELISA-titer |
| Mumps vaccine 20 μg | 5 μg | 0.074 |
| Control 20 μg | 0 μg | 0.023 |

Example 18

Mixed vaccine of measles, rubella and mumps-LTB (intranasal) :

Mixed vaccine of measles, rubella and mumps-LTB intranasal preparation was prepared by mixing the mixed vaccine with LTB dissolved in PBS and filtrated aseptically, preparing a mixture (20 μℓ) of virus particles corresponding to measles vaccine (7 μg), rubella vaccine (1 μg) and mumps vaccine (7 μg), and LTB (5 μg), added stabilizer, and filled in a bottle.

The preparation is stored in a dark-cool place

below 10℃.

The vaccine with or without LTB was inoculated 2 times in mice with 3 weeks interval, and measured the serum antibody production.

ELISA antibody titer administered the vaccine alone was 0.18, 0.07 and 0.13, respectively for measles , rubella and mumps, and that of LTB added vaccine was 0.34, 0.27 and 0.28, respectively. Augmentation of antibody production was observed when administered vaccine with LTB.

Table : Augmentation of antibody production by an addition of LTB in a mixed vaccine of measles, rubella and mumps

| Inoculum size | | Antibody production ELISA-titer |
|---|---|---|
| Antigen | LTB | |
| Measles vaccine 7 μg | 5 μg | Measles 0.34 |
| Rubella vaccine 1 μg | | Rubella 0.27 |
| Mumps vaccine 7 μg | | Mumps 0.28 |
| Control | 0 μg | Measles 0.18 |
| | | Rubella 0.07 |
| | | Mumps 0.13 |

Example 19

Rota vaccine-LTB (Peroral and intranasal) :

Rota vaccine-LTB oral and intranasal preparation was prepared by mixing rota vaccine with LTB dissolved in PBS

and filtrated aseptically, preparing a mixture (20 $\mu\ell$) of virus particles corresponding to rota vaccine (3.3 $\mu$g) and LTB (5 $\mu$g), added stabilizer, and filled in a bottle.

The preparation is stored in a dark-cool place below 10℃.

Rota vaccine with or without LTB was inoculated 2 times in mice with 3 weeks interval, and measured the serum antibody production.

ELISA antibody titer administered the vaccine alone was 0.063 for intranasal administration, and that of LTB added vaccine was 0.348 and 0.024 for oral administration and 0.177 for LTB added vaccine with oral administration.

Augmentation of antibody production was observed when administered vaccine with LTB.

Table : Augmentation of antibody production by an addition of LTB in rota vaccine

| Inoculum size | | | | | Antibody production ELISA-titer |
|---|---|---|---|---|---|
| Antigen | | | | LTB | |
| Rota vaccine | Intranasal | Vaccine | 3.3 μg | 5 μg | 0.348 |
| | | Control | 3.3 μg | 0 μg | 0.063 |
| | Oral | Vaccine | 3.3 μg | 5 μg | 0.177 |
| | | Control | 3.3 μg | 0 μg | 0.024 |

Example 20

Mycoplasma vaccine-LTB (injection) :

Mycoplasma vaccine-LTB injectable preparation was prepared by mixing mycoplasma vaccine with LTB dissolved in PBS and filtered aseptically, preparing a mixture (1 mℓ) of mycoplasma corresponding to vaccine ($2.0 \times 10^{10}$ CFU) (colony forming unit) and LTB (10 μg), added stabilizer, and filled in a vial.

The preparation is stored in a dark-cool place below 10℃.

The vaccine with or without LTB was inoculated 3 times in chicken with 2 weeks interval, and observed the number of lesions after challenge of mycoplasma infection.

The administration of LTB added vaccine showed marked protective effect as compared with that of the vaccine alone.

Table : Decrease of lesion after challenge of mycoplasma infection administered mycoplasma vaccine with LTB

| Inoculum size | | Lesions | |
|---|---|---|---|
| Antigen | LTB | | |
| total mycoplasma cells*** $1.0 \times 10^{10}$ CFU | 5 μg | * 3／12 | ** 1.23 |
| Ultrasonication $1.0 \times 10^{10}$ CFU | 5 μg | 2／11 | 1.27 |
| Control $1.0 \times 10^{10}$ CFU | 0 μg | 10／10 | 2.77 |

* : No. of animals showing lesion / No. of animals used

** : Mean of lesion / gorup

*** : CFU

BRIEF EXPLANATION OF DRAWINGS :

Fig. 1A ~ 1D : Time course of primary antibody production administering a vaccine of the present invention; and

Fig. 2 : No. of infections lesions in lung after administering a vaccine of the present invention.

CLAIMS

1. A vaccine preparation comprising a vaccine and a toxin or a subunit thereof.

2. A preparation according to claim 1 wherein the toxin is a bacterial toxin.

3. A preparation according to claim 2 wherein the bacterial toxin is cholera toxin, Staphylococcal -hemolysin, Staphylococcal -hemolysin, Vibrio thermostable direct hemolysin, pertussis toxin or E. coli heat-labile toxin.

4. A preparation according to any one of claims 1 to 3 wherein the subunit of the toxin is a B subunit or a part thereof.

5. A preparation according to any one of claims 1 to 4 wherein the vaccine is influenza vaccine, pertussis vaccine, Japanese encephalitis vaccine, a mixed vaccine of pertussis vaccine, diphtheria and tetanus toxoid, hepatitis B vaccine, Rota vaccine, measles vaccine, rubella vaccine, mumps vaccine, a mixed vaccine for measles, rubella and mumps or mycoplasma vaccine.

6. A preparation according to any one of claims 1 to 5 wherein the ratio of the vaccine to the toxin or subunit thereof is from 1:0.0001 to 1:10,000 (w/v).

7. A preparation according to any one of claims 1 to 6 which is an intranasal vaccine preparation.

8. A preparation according to any one of claims 1 to 7 which is in injectable form, spray form or a form suitable for oral administration.

9. A vaccine preparation as defined in any one of claims 1 to 8 for use in a method of treatment of the human or animal body by therapy.

Published 1989 at The Patent Office, State House, 66/71 High Holborn, London WC1R 4TP. Further copies may be obtained from The Patent Office. Sales Branch, St Mary Cray, Orpington, Kent BR5 3RD. Printed by Multiplex techniques ltd, St Mary Cray, Kent, Con. 1/87